CHAPTER 4

DRMs exist in sphingolipid- and cholesterol-depleted neuroblastoma cells. Impact on DRM localisation and function of MRP1

Anne-Jan Dijkhuis[1,2], Karin Klappe[1], Anneke de Boer[1], Anne-Marije Huls[1], Hjalmar Permentier[4], Andries Bruins[4], Willem Kamps[2], Hannie Sietsma[3], Jan Willem Kok[1]

[1]Department of Cell Biology, Section Membrane Cell Biology, University Medical Centre Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[2]Department of Paediatric Oncology and Haematology, Beatrix Children's Hospital, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands
[3]Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands
[4]Centre for Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands.

Introduction

Lipid rafts have been established as an important feature in cell membranes with functions in signal transduction, cell adhesion and protein sorting (Pike, 2003). The physical properties of these membrane microdomains are distinct from those of the surrounding membrane. This is generally considered to be the result of a different lipid composition in these domains compared to the surrounding membrane. Membrane microdomains are enriched in sphingolipids with a high degree of saturation in their fatty acyl chains and cholesterol. The presence of these lipids is thought to stabilise membrane domains, due to the ability of saturated sphingolipids and cholesterol to tightly pack in the plane of the membrane (Brown and London, 2000; Brown, 2002). In model membranes cholesterol induces tight packing of sphingolipids into a liquid-ordered (L_o) state. It is not known how membrane microdomains arise in cells, but it is likely that this process starts with small scale protein-lipid interactions and size increase may occur due to protein-protein interactions (Pike, 2004). Lipid rafts in cells are operationally defined as complexes of molecules that are insoluble at low temperature in Triton X-100 or other detergents, such as Lubrol (London and Brown, 2000; Schuck et al., 2003). We refer to these isolated lipid rafts as detergent-resistant membranes (DRMs). Most studies on the occurrence, properties and functions of lipid rafts in cells rely on the use of detergents for their isolation, although detergent-free isolation methods have also been developed (Macdonald and Pike, 2005).

ATP-binding cassette (ABC) transporter proteins, such as P-glycoprotein (Pgp) and multidrug resistance-related protein (MRP1) have been associated with DRMs. Originally Pgp was localised in caveolae (Lavie et al., 1998; Demeule et al., 2000), but later studies showed localisation of both Pgp and MRP1 in non-caveolar DRMs (Hinrichs et al., 2004; Radeva et al., 2005). Both ABC transporters were more strongly enriched in Lubrol-based DRMs compared to Triton X-100-based DRMs (Hinrichs et al., 2004). Given their localisation in DRMs, the function of ABC transporters may well be dependent on or modulated by sphingolipids and/or cholesterol. Indeed, some evidence exists for modulation of Pgp function by cholesterol and involvement of DRMs in this process (Luker et al., 2000; Troost et al., 2004). Concerning a role for sphingolipids in modulation of ABC transporter function, several hypotheses exist but a coherent picture has not yet emerged. In multidrug resistant (MDR) cells over-expressing Pgp or MRP1, several changes in sphingolipid metabolism have been observed, including accumulation of glucosylceramide (GlcCer). The latter has been related to increased glucosylceramide synthase (GCS) activity (Bleicher and

Cabot, 2002). Because GCS is responsible for metabolic removal of ceramide (Cer) from the sphingolipid pool, an increased activity of this enzyme is beneficial to tumour cells that are under stress of cytostatics or other stress factors that induce Cer formation and subsequent apoptosis. On the other hand, enhanced GlcCer formation may be part of a more comprehensive response involving up regulation of the total glycolipid pool, including gangliosides. The latter may be involved in MDR, either related to or independent of ABC transporters (Sietsma et al., 2001). One study described activation of Pgp activity by gangliosides through modulation of its phosphorylation state (Plo et al., 2002). Finally, an intimate relationship between sphingolipids and ABC transporters is inherent to the flippase activity of the latter for which sphingolipids can function as substrates (Eckford and Sharom, 2005).

In this study we investigated the role of sphingolipids and cholesterol in the integrity of DRMs in neuroblastoma and show that both types of lipids can be strongly depleted in cells and also in DRMs without abrogating the ability to isolate DRMs employing Lubrol. DRM integrity was confirmed by protein content and gradient distribution of classical DRM markers Src and caveolin-1 (Cav-1). Sphingolipid depletion affected neither MRP1 localisation in DRMs nor MRP1 efflux function. On the other hand cholesterol depletion affected both, causing MRP1 to shift in gradients out of DRM fractions and reducing its efflux activity. We conclude that sphingolipids are not as relevant to DRM integrity as generally thought and are not relevant for MRP1 activity. On the contrary, cholesterol does modulate MRP1 activity and appears to do so in the context of membrane domains.

Materials and methods

Materials

MK571 was a gift from Prof. A.W. Ford-Hutchinson (Merck-Frosst, Inc., Kirkland,Canada). All cell culture plastic was from Costar (Cambridge, MA). Dulbecco's modified Eagle medium, Hank's balanced salt solution (HBSS), antibiotics, L-glutamine, sodium pyruvate and trypsin were from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). L-[U-^{14}C]serine was purchased from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK). HPTLC plates were from Merck (Amsterdam, The Netherlands). C12-fatty acid homologues of Cer, sphingomyelin (SM), glucosylceramide (GlcCer) and lactosylceramide (LacCer) were from Avanti Polar Lipids (Alabaster, AL, USA). 5-carboxyfluorescein diacetate (CFDA), 3-[4,5-dimethylthiazol-2-yl]-

2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, lovastatin and methyl-β-cyclodextrin (M-β-CD) were from Sigma-Aldrich (St. Louis, MO, USA). Lubrol was obtained from Serva (Heidelberg, Germany). ISP-1 was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The rat monoclonal anti-MRP1 (MRPr1) antibody was obtained from Signet Laboratories (Dedham, MD, USA). The polyclonal rabbit anti-Cav-1 antibody was from Transduction Laboratories (Lexington, KY, USA). The polyclonal rabbit anti-c-Src antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture

The murine neuroblastoma cell line Neuro-2a was purchased from the ATCC (Manassas, VA, USA). The cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate, under standard incubator conditions (humidified atmosphere, 5% CO2, 37°C). In order to deplete sphingolipid content, cells were incubated in the presence of 0.5 µM ISP-1 for three days, unless stated otherwise. In order to deplete cholesterol content, cells were incubated in the presence of 1µg/ml lovastatin for 24h and/or 10 mM M-β-CD for 1h in serum-free medium, unless stated otherwise.

Equilibrium radiolabelling and analysis of cellular sphingolipids

Sphingolipid pools were metabolically radiolabelled by growing the cells for 72h in the presence of L-[U-14C]serine (0.5 µCi/ml), a precursor molecule for sphingolipid biosynthesis. Cells were harvested by scraping and centrifuged, followed by lipid extraction from the cell pellet (Bligh and Dyer, 1959). Aliquots of the lipid extracts were taken for determination of the total amount of lipid-incorporated radioactivity. Acylglycerolipids were hydrolysed during a 1h incubation at 37°C in $CHCl_3/CH_3OH$ (1:1, v/v) containing NaOH (0.1 M). The remaining lipids were re-extracted and applied on HPTLC plates. Plates were developed in $CHCl_3/CH_3OH/H_2O$ (14:6:1, v/v/v) in the first dimension. Plates were then sprayed with 2.5% H_3BO_3 (w/v) in CH_3OH and developed in $CHCl_3/CH_3OH$/25% (w/v) NH_4OH (13:7:1, v/v/v) in the second dimension. After autoradiography, GlcCer, LacCer and SM containing spots were identified with the aid of standards and scraped from the plates. Plates were then developed in the second dimension, but now in reversed direction, in $CHCl_3/CH_3COOH$ (9:1, v/v). Plates were dried and, after staining in I_2 vapour, Cer containing spots were scraped. Radioactivity was measured by scintillation counting (Packard Topcount microplate

scintillation counter, Meriden, CT). Lipid levels were expressed as dps incorporated in a specific lipid species per 10^3 dps of total lipid-incorporated radioactivity.

Isolation of DRMs

DRM fractions were isolated from cells as described (Lisanti et al., 1995). For each isolation, confluent cells from two 75 cm^2 flasks were washed once with HBSS, harvested by scraping in 2 ml of ice-cold Tris-NaCl-EDTA buffer (TNE) (20 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and protease inhibitors) containing 0.5% (w/v) Lubrol and vortexed. After 30 min incubation on ice, cells were homogenised further by passing the lysate at least ten times through a 21 Gauge needle. Two ml of the lysate was transferred to a centrifuge tube and mixed with 2 ml of 80% (w/v) sucrose in TNE. On top of this, 4 ml of 35% (w/v) and 3 ml of 5% (w/v) sucrose in TNE were successively loaded, resulting in a discontinuous gradient. Gradients were centrifuged in a Beckman SW41 swing-out rotor (Beckman Coulter, Inc., Fullerton, CA, USA) at 40,000 rpm for 18-20h at 4°C. Eleven fractions of 1 ml each were collected (from top to bottom), vortexed and stored at -80°C. The protein content (Smith et al., 1985) of all fractions was measured using bovine serum albumin as standard.

Liquid chromatography-electrospray tandem mass spectrometry

Sphingolipids were extracted and analysed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) on a PE-Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ionspray source as described previously (Sullards and Merrill, 2001). HPLC separation was performed as described previously (Sullards et al, 2003), with the following changes: an APS-2 Hypersil 150x2.1 mm column (Thermo Electron, Breda, The Netherlands) was used and the flow rate was 200 µl/min. N_2 was used as the nebulising gas and drying gas for the turbo ionspray source. The ion spray needle was held at 5,500 V, and the orifice and ring voltages were kept low (30 and 150 V, respectively) to prevent collisional decomposition of molecular ions before entry into the first quadrupole; the orifice temperature was set to 500°C. N_2 was used to collisionally induce dissociations in Q2. Multiple reaction monitoring scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. MRM transitions and collision energies for each species were taken from table 1 in Sullards et al. (2003). The transitions correspond to ceramides, glucosylceramides, lactosylceramides and sphingomyelins with a d18:1 sphingoid base (sphingosine) and C16:0, DHC16:0, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, and C26:0 fatty acids, respectively. Quantitation was

achieved by spiking the samples before extraction with the C12-fatty acid homologues of Cer, SM, LacCer and GlcCer.

Cholesterol and phosphate determination on cell lysate and DRMs

Lubrol-based cell lysates were prepared and part of this lysate was used to isolate DRMs (see "Isolation of DRMs"). The DRM fractions were pooled. After a protein determination (Smith et al., 1985) on both the lysate and the pooled DRM fractions, lipids were extracted (Bligh and Dyer, 1959). In the extract the cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase/peroxidase assay (Gamble et al., 1978). The phosphorus content, as a measure for the phospholipid content in the lysate and the pooled DRM fractions, was determined by a phosphate assay (Böttcher et al., 1961).

Immunoblot analysis

Protein from the gradient was TCA-precipitated and resuspended in sample buffer. TCA-precipitated proteins were resolved on SDS-PAGE (10%) minigels and subsequently electrotransferred onto a nitrocellulose membrane (Trans-Blot Transfer Medium membrane, Bio-Rad, Hercules, CA, USA). The membranes were rinsed with PBS and incubated (1-2h, RT) with 5% (w/v) non-fat dry milk in PBS. Membranes were rinsed in washing buffer (PBS containing 0.3% (v/v) Tween 20) and incubated (at least 2h, RT) with a primary antibody against MRP1 (1:500), Cav-1 (1:1000) or Src (1:1000) in washing buffer containing 0.5% (w/v) non-fat dry milk. Membranes were rinsed in washing buffer and subsequently incubated for 2h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000) (ECL, Amersham Biosciences UK, Buckinghamshire, UK) in washing buffer containing 0.5% (w/v) non-fat dry milk (2h, RT). Membranes were incubated in chemiluminescence substrate solution (ECL, Amersham Biosciences), according to the manufacturer's instructions, and immunoreactive complexes were visualised by exposure to a Konica Minolta medical film (Tokyo, Japan).

Detection of MRP1-mediated efflux by FACS analysis

Cholesterol-treated Neuro-2a cells were allowed to recover for 1h in serum-free medium prior to harvesting. Cells ($0.5x10^6$ in HBSS), which were harvested by trypsinisation, were incubated with the MRP1 substrate CFDA (0.5 µM, unless stated otherwise) at 10°C for 60 min. Cells were washed twice with ice-cold HBSS and incubated in the presence or absence of the MRP1 inhibitor MK571 (20 µM) at 37°C for during various time intervals. Efflux of

fluorescent substrate was stopped by washing cells with ice-cold buffer, followed by resuspension in buffer containing MK571. Retention of fluorescence was determined by flow cytometric analysis using an Elite^{TM} flow cytometer (Beckman Coulter, Miami, FL). For each sample 10000 events were collected and analysed using Win-list 5.0 software (Verity Software House Inc., Topsham, ME).

Measurement of cellular sensitivity to cytotoxic drugs (MTT assay)

One thousand cells/well were plated in microtiter plates. For depletion of cholesterol, cells were washed 24h after plating with serum-free medium and incubated in the presence of 10 mM M-β-CD in serum-free medium for 1h at 37°C. Subsequently, cells were washed with serum-free medium and incubated for 48h in the presence of 1μg/ml lovastatin in serum-containing medium. For sphingolipid depletion, cells were pre-incubated with ISP-1 (0.5 μM) for three days, subsequently trypsinised and plated (one thousand cells/well). Seventy-two hours after plating viable cells were determined as previously described (Carmichael et al., 1987). Briefly, 100 μg MTT was added to each well and cells were incubated for 2h at 37°C. Plates were then centrifuged (15 min, 900×g) and the supernatants were removed. Pellets were dissolved in DMSO and absorbencies were measured in a microtiter plate reader (μQuant, Bio-Tek Instruments, Winooski, VT, USA) at a λ of 570 nm. The background absorbency was subtracted from all values and data were expressed as percentage compared to untreated control cells (=100%).

Results

Efficient depletion of sphingolipid and cholesterol content

The SPT inhibitor ISP-1 (0.5μM) efficiently depleted sphingolipid content in Neuro-2a cells upon a three-day incubation. The pool of sphingolipids was reduced by 88% ± 5 (n=3), as determined by equilibrium radiolabelling. Depletion was highly efficient for Cer and glycosphingolipids and slightly less for SM (Table I). Measurement of endogenous sphingolipid mass using liquid chromatography-electrospray tandem mass spectrometry showed similar results. Levels of Cer and glycosphingolipids were depleted by at least 90% in whole cells (Table I). SM depletion again was slightly less efficient (87%).

M-β-CD and lovastatin were used to deplete cholesterol from Neuro-2a cells. With M-β-CD alone depletion was about 82% in whole cells and 88% in Lubrol-based DRMs. Lovastatin alone reduced cholesterol content to 50 % (n=2) and the combined use of M-β-CD

and lovastatin did not result in further reduced levels of cholesterol compared to M-β-CD alone treatment (data not shown).

The depletion of sphingolipids using ISP-1 or the depletion of cholesterol using M-β-CD did not significantly affect cell viability, as determined by the MTT assay (ISP-1-treated cells: 88,4% ± 16,8% and M-β-CD-treated cells: 81,4% ± 18,6% cell viability compared to control (100%) cells).

Table I. Sphingolipid and cholesterol depletion in Neuro-2a cells by ISP-1 and M-β-CD, respectively

A	**Sphingolipid depletion (ISP-1)**		
	Radiolabelling Whole cells (%)	LC-ESI-MS/MS Whole cells (%)	LC-ESI-MS/MS Lubrol-based DRMs (%)
Cer	3.1 ± 2.2	6.5 ± 1.5	5.8 ± 4.0
GlcCer	nd	4.3 ± 0.2	7.9 ± 3.6
LacCer	nd	1.5 ± 0.5	2.0 ± 1.2
SM	11.8 ± 4.0	12.9 ± 1.3	21.5 ± 11.4
B	**Cholesterol depletion (M-β-CD)**		
	Whole cells (%)		Lubrol-based DRMs (%)
Cholesterol	17.9 ± 2.2		11.7 ± 5.7

A) Neuro-2a cells were incubated in the presence or absence of ISP-1 (0.5 μM) for three days. In order to radiolabel sphingolipids, cells were also incubated in the presence of L-[U-^{14}C]serine during this period. Sphingolipids were extracted and quantified using scintillation counting or liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS). ISP-1 treatment reduced sphingolipid content to about 10-20% in whole cells as well as Lubrol-based DRMs. Values of each lipid are expressed as a percentage of untreated cells (100%). Data represent the mean ± S.D. of 3 independent experiments. nd: not detectable

B) Neuro-2a cells were incubated in the presence or absence of M-β-CD (10 mM) for one hour. Cholesterol was extracted and quantified. M-β-CD reduced cholesterol content to about 10-20% of that of untreated cells (100%) in whole cells as well as Lubrol-based DRMs. Data represent the mean ± S.D. of three independent experiments.

Depletion of sphingolipid or cholesterol does not abrogate Lubrol-based DRM isolation

Surprisingly, when Neuro-2a cells were efficiently depleted of either sphingolipids or cholesterol, we were still able to isolate Lubrol-based DRMs from these cells. Moreover, the protein content of these DRMs, i.e. the protein profile of the sucrose gradient fractions was identical to that of control cells (Fig. 1A). Also the gradient distributions of established DRM protein markers Cav-1 and Src were indistinguishable between ISP-1-treated, M-β-CD-treated and control cells (Fig. 1B,C). This raised the question whether sphingolipid and cholesterol depletion, respectively, actually diminished the DRM-associated lipid pools. However, in

isolated Lubrol-based DRMs (Table I), Cer and glycosphingolipids were also depleted by at least 90% and SM again somewhat less efficient (79%). On average, residual levels of sphingolipids in whole cells were around 8% and in Lubrol-based DRMs around 15% (Table I).

We conclude that efficient depletion of either sphingolipid or cholesterol did not hamper the potential to isolate DRMs from Neuro-2a cells. These DRMs, although themselves efficiently depleted of sphingolipids or cholesterol, did not appear to differ in protein profile compared to DRMs from control cells.

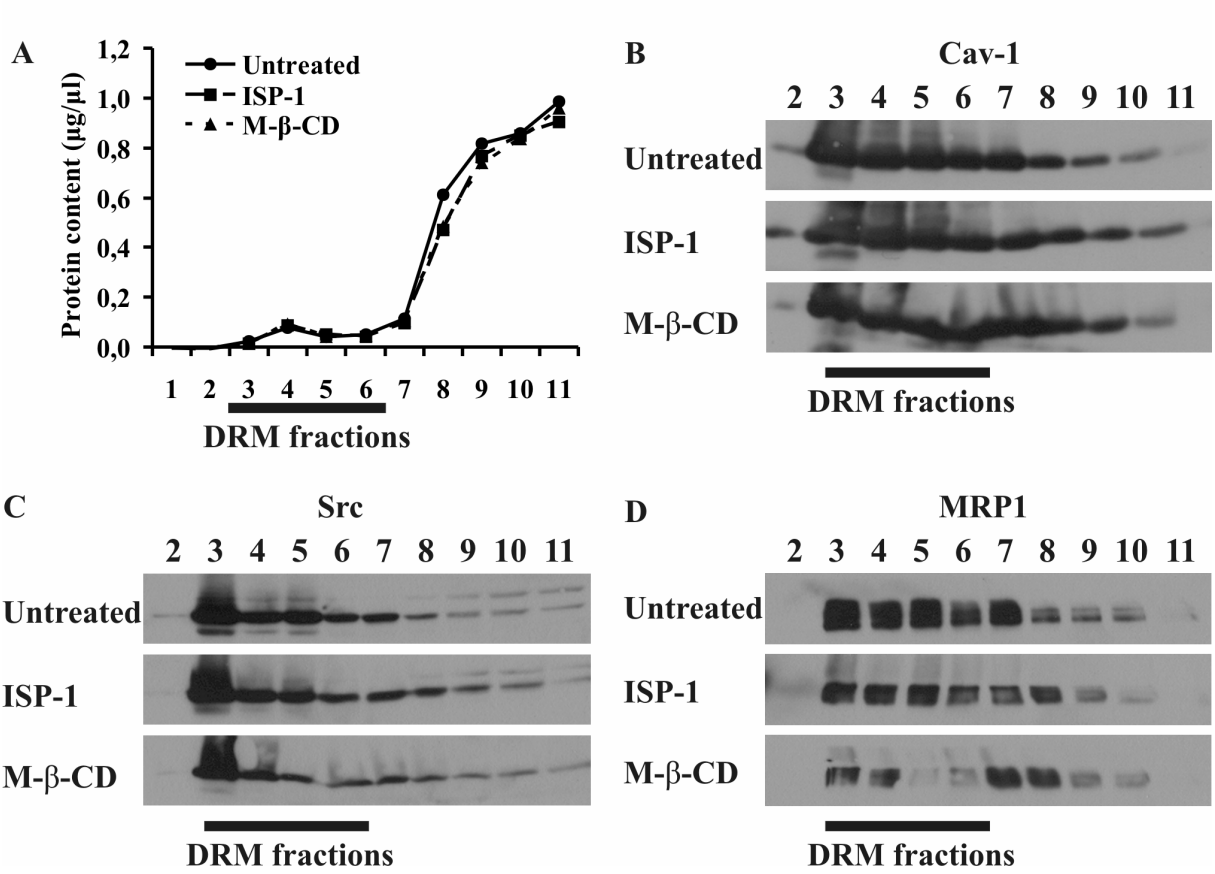

Figure 1. Effects of sphingolipid or cholesterol depletion on DRMs and protein marker distribution

A. The protein profile on sucrose gradients of Lubrol-based DRMs is not affected by ISP-1 or M-β-CD treatment. Lubrol lysates were fractionated by flotation in a discontinuous sucrose density gradient. The protein content of each fraction was determined.

B-D. The sucrose gradient distributions of Cav-1 (B), Src (C) and MRP1 (D) are not affected by ISP-1 treatment. Only the MRP1 distribution is changed by M-β-CD treatment, which causes a shift of MRP1 from DRM fractions to higher density fractions. Lubrol lysates were fractionated by flotation in a discontinuous sucrose density gradient. Aliquots of each fraction, containing equal protein levels, were subjected to SDS-PAGE and immunoblotting.

Depletion of the total sphingolipid pool does not affect MRP1 efflux activity

We next determined whether depletion of sphingolipids or cholesterol affected DRM localisation and function of a specific protein, i.e. MRP1. This protein was chosen in view of its relevance to MDR, its association with (Lubrol-based) DRMs and the potential modulation of its activity by sphingolipids and/or cholesterol. To study its function, we tested MRP1 efflux activity. In order to determine MRP1 efflux activity, untreated and ISP-1-treated Neuro-2a cells were loaded with CFDA, which is a fluorescent substrate of MRP1. The use of 0.5 μM CFDA to load the cells at 10°C resulted in a higher intracellular CFDA concentration in ISP-1-treated cells compared to untreated cells (data not shown). Therefore, ISP-1-treated cells were loaded with a lower CFDA concentration (0.3 μM), such that intracellular CFDA concentrations in untreated and ISP-1-treated cells were the same after loading. Efflux activity was determined on the basis of fluorescence retention after cells were incubated at 37°C in the presence or absence of the MRP1 inhibitor MK571. MRP1 efflux activity was very similar in ISP-1-treated cells compared to control cells (Fig. 2). Thus, depletion of sphingolipids from Neuro-2a cells using ISP-1 did not affect MRP1 efflux activity. In accordance with the absence of an effect of sphingolipid depletion on MRP1 efflux function, there was no effect on the DRM localisation of the ABC transporter, as indicated by a very similar profile on sucrose gradients (Fig. 1D).

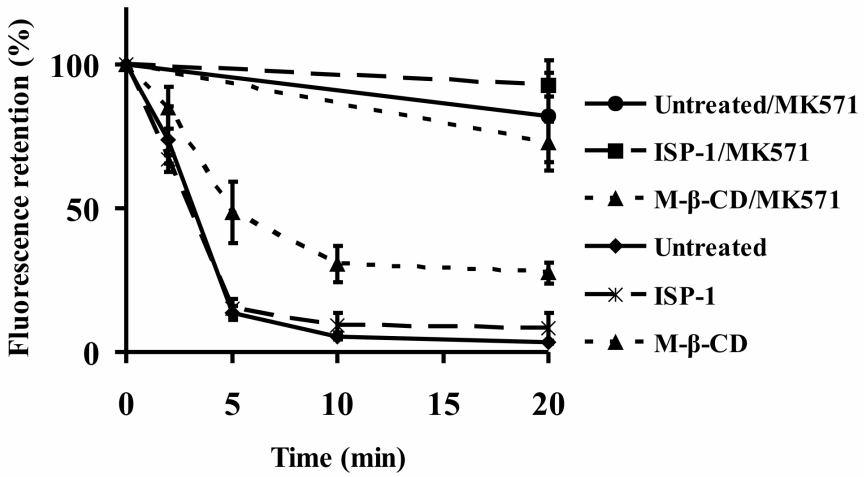

Figure 2. Efflux activity of MRP1 is affected by M-β-CD, but not by ISP-1 treatment
After M-β-CD or ISP-1 treatment, Neuro-2a cells were loaded with CFDA (0.5 or 0.3 μM, see text for details). Retention of fluorescence was determined by cytometric analysis at several time-points after cells were placed at 37°C in the presence (+) or absence (-) of MK571 (20 μM). The data show that cholesterol depletion lowers MRP1-mediated efflux activity, while sphingolipid depletion is without effect. The cholesterol depletion effect on MRP1-mediated efflux is partial compared to the effect of the established MRP1 inhibitor, MK571.

Cholesterol depletion reduces MRP1 efflux activity and alters MRP1 raft localisation

In contrast to sphingolipid depletion, efficient depletion of cholesterol did show effects on DRM localisation of MRP1 as well as its function. Also here differences in influx between cholesterol-depleted and control cells were corrected by loading M-β-CD-treated cells with a lower CFDA concentration (0.3 μM), such that intracellular CFDA concentrations in untreated and M-β-CD -treated cells were the same after loading. Cholesterol depletion with M-β-CD resulted in a partial but significant decrease in MRP1 efflux activity (Fig. 2). Concomitant with this partial effect on MRP1 function, the ABC transporter partly shifted out of DRM fractions to higher density fractions in sucrose gradients (Fig. 1D).

Discussion

Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and sphingolipids. They appear to be small in size, but together may constitute a relatively large fraction of the plasma membrane (Harder and Simons, 1997; Simons and Ikonen, 1997). The high content of glycosphingolipids and sphingomyelin in DRMs gave rise to two different models for lipid raft formation. The first model points out the importance of the relative long length and high saturation of the acyl chains of glycosphingolipids and sphingomyelin for raft formation. This allows close packing of the lipids resulting in a high melting temperature (T_m). Self-aggregates of sphingolipids form a separate phase that is less fluid (liquid-ordered) than the bulk liquid-disordered phospholipids. Cholesterol is recruited to these aggregates, due to its ability to pack tightly with lipids of high T_m (Brown and London, 2000; Brown, 2002). According to the second model, lipid rafts are primarily clusters of glycosphingolipids and sphingomyelin held together through hydrogen-bonding between glycosphingolipid head groups and close packing of the sphingolipids. Cholesterol fills up the gaps between the bulky-heads of the glycosphingolipids (Simons and Ikonen, 1997).

Interestingly, although glycosphingolipids are enriched in DRMs they do not appear to be essential for the formation of these membrane domains. It was shown that glycosphingolipid-deficient GM95 melanoma cells had similar amounts of DRMs compared to control cells. The loss of glycosphingolipid mass in these cells due to mutation of the gene encoding GCS was exactly compensated by an increase in SM mass (Hidari et al, 1996; van Riezen, M., Kok, J.W. and Merrill, A.H., jr., unpublished observations). Glycosphingolipids in DRMs of GM95 cells had been substituted by SM (Ostermeyer et al., 1999). However,

glycosphingolipids were essential for Src kinase association to DRMs and hence appear to be essential for functional properties of rafts (Inokuchi et al., 2000). In this study we show for the first time that even under conditions when both glycosphingolipids and SM, in fact when almost all sphingolipids are depleted from Neuro-2a cells during long-term ISP-1 treatment, these cells still have similar amounts of DRMs compared to control cells. Moreover, also the other important lipid constituent of DRMs, i.e. cholesterol, appears to be largely dispensable in this respect, as similar amounts of DRMs were isolated after efficient short-term M-β-CD treatment. This leads to the conclusion that DRMs can be isolated from cells which are severely depleted in either sphingolipids or cholesterol, considered the two most important lipid constituents of lipid rafts. Hence, sphingolipids do not appear to be essential for the formation while both sphingolipids and cholesterol do not appear to be essential for the integrity of lipid rafts as defined by detergent-isolation.

One of the best characterised MDR mechanisms is the over-expression of energy-dependent drug efflux proteins, which prevent intracellular drug accumulation. Of these proteins, all members of the ABC transporter protein super family, Pgp (or ABC B1) and MRP1 (or ABC C1) are the most widely studied. Both ABC transporters are known to depend on their direct lipid environment for optimal functioning (Dudeja et al., 1995; Sinicrope et al., 1992). Upon reconstitution in model membranes, their ATPase activity is dependent on the close proximity of specific phospholipids, especially phosphatidylethanolamine and phosphatidylserine (Doige and Sharom, 1993; Romsicki and Sharom, 1998; Chang et al., 1997; Mao et al., 2000). Furthermore, P-glycoprotein was found to have a higher affinity for its substrates when the surrounding lipids are in gel phase rather than in liquid-crystalline phase (Romsicki and Sharom, 1999). This gel phase occurs when lipids have a high degree of saturation, like sphingolipids, which enables them to pack tightly. This is also an important characteristic of membrane microdomains or lipid rafts, including caveolae (Brown and London, 2000; Schroeder et al., 1994).

Lavie et al. (1998) have shown for the first time the association of an ABC transporter with a membrane domain. They found that a substantial fraction of Pgp was located in Cav-1 containing Triton X-100-based DRMs in Pgp over expressing cells. More evidence for membrane domain association of ABC transporters, and its functional implication came from cholesterol depletion experiments. Cholesterol depletion not only resulted in a shift of P-glycoprotein out of DRM fractions, but P-glycoprotein-mediated drug transport was also affected (Luker et al., 2000). In Caco-2 cell monolayers, cholesterol depletion significantly impaired the efflux activity of both Pgp and MRP2 (Yunomae et al., 2003). Pgp association to

caveolae and Pgp-substrate levels were also found to be correlated (Demeule et al., 2000). On the other hand, it was shown that Pgp and MRP1 were not associated with caveolae in two human MDR tumour cell lines (Hinrichs et al., 2004). Both MRP1 and Pgp were found to be enriched in membrane domains defined by their insolubility in the non-ionic detergent Lubrol. In 2780AD cells, which do not express Cav-1 and hence lack caveolae, Pgp was located in non-caveolar DRMs. $HT29^{col}$ cells do express Cav-1, but MRP1 and Cav-1 did not co-localise and were not co-immunoprecipitated (Hinrichs et al., 2004). Hence, it appears unlikely that Cav-1 or caveolae play a significant role in the accommodation or function of ATP-binding cassette transporters. A recent study arrived to the same conclusion regarding dissociation of Pgp and caveolae in a MDR CHO cell line and postulated that Pgp resides in an intermediate-density membrane microdomain which is distinct from both caveolar domains and classical lipid rafts, the latter defined by Triton X-100 insolubility and presence of GM1. These Pgp-containing domains were defined by insolubility in Brij-96 (Radeva et al., 2005).

It was previously shown that inhibition of GCS and hence depletion of glycosphingolipids did not affect MRP1 efflux function in $HT29^{col}$ MDR tumour cells (Klappe et al., 2004). Moreover, in neuroblastoma cells GCS inhibition neither affected Pgp nor MRP1 function (Dijkhuis et al., 2006b). Here we show for the first time that depletion of all sphingolipid classes, including Cer and SM, did not affect MRP1 efflux function. On the other hand, cholesterol depletion did result in a reduction of MRP1 efflux activity and a concomitant shift of MRP1 from DRM fractions in sucrose gradients to higher density fractions. The latter is reminiscent of the shift of Pgp observed in CHO cells upon cholesterol depletion (Radeva et al., 2005). This indicates that although DRMs retain their integrity upon cholesterol depletion, the properties of these DRMs change and these changes likely result in an altered localisation and possibly an altered activity of the DRM-associated protein MRP1. These results are in line with a recent analysis of the lipid composition of Lubrol- and Triton X-100-based DRMs. ABC transporter-containing Lubrol-based DRMs were shown to be enriched in cholesterol and sphingolipids. However, sphingolipids were less enriched in Lubrol-based DRMs compared to Triton X-100-based DRMs. Instead, Lubrol-based DRMs contained relatively large amounts of the phospholipids phosphatidylethanolamine and phosphatidylserine (Hinrichs et al, 2005b). A layered raft model was proposed in which Lubrol-based DRMs consist of a highly sphingolipid-enriched Triton X-100 insoluble core, surrounded by a Triton X-100 soluble region, which contains relatively high levels of cholesterol and specific aminophospholipids and harbours most of the DRM-associated ABC transporter molecules (Hinrichs et al., 2005a). Thus, in the ABC-transporter-containing

subdomains cholesterol appears to play an important role, in conjunction with (specific) phospholipids, whereas sphingolipids are more enriched in ABC-transporter-poor subdomains of DRMs and hence do not impact on ABC-transporter function.

In our view, this study has the following implications: *a*) the definition of rafts that was very recently reached in a specialised membrane raft meeting (Pike, 2006) may already have to be reconsidered. The definition of rafts that was reached states: 'Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalise cellular processes. Small rafts can sometimes be stabilised to form larger platforms through protein-protein and protein-lipid interactions' (Pike, 2006). However, our observations indicate that DRMs can be isolated from sphingolipid or cholesterol depleted cells. Future studies in other cell lines employing various membrane domain isolation techniques should confirm this conclusion and show whether this is a ubiquitous phenomenon. In addition, it will be important to establish the characteristics of sphingolipid- and cholesterol-poor membrane rafts, which discriminate rafts from the surrounding membrane. *b*) We can conclude from our study that sphingolipids are not essential to regulation of ABC transporter activity and do not appear to represent a universal target for therapeutic potential. On the other hand, modulation of cholesterol levels in tumour cells appears to be a strategy to manipulate ABC transporter activity and thus could be considered a basis for future treatment of neuroblastoma. From a mechanistic point of view, it is important to establish whether the effects of cholesterol on ABC transporter function are indeed membrane raft-mediated, as our results suggest, or alternatively a consequence of direct cholesterol-ABC transporter interaction. From our study we can therefore not conclude whether or not membrane rafts as such are implicated in MDR and constitute a potential target for therapeutic intervention.

Abstract

We show that extensive depletion of sphingolipids or cholesterol in neuroblastoma cells does not abrogate the ability to isolate Lubrol-based detergent-resistant membranes (DRMs) from these cells. DRM fractions of these cells are strongly depleted of sphingolipids or cholesterol but contain equal amounts of protein compared to DRMs of control cells. Moreover, classical DRM protein markers Src and caveolin-1 (Cav-1) display a normal gradient distribution in sphingolipid- or cholesterol-depleted cells. We conclude that DRMs can be isolated from cells with very low sphingolipid or cholesterol levels and these DRMs are themselves severely depleted of sphingolipids and cholesterol, respectively. To study functional consequences of lipid depletion, the DRM localisation and efflux function of MRP1 was investigated. Sphingolipid depletion affected neither DRM localisation nor efflux function of multidrug resistance-related protein (MRP1). On the contrary, cholesterol depletion caused a partial shift of MRP1 from DRM fractions to higher density gradient fractions and also resulted in a partial inhibition of MRP1 efflux function. We conclude that in contrast to sphingolipids, cholesterol affects MRP1 function in neuroblastoma cells and this may be related to its localisation in DRMs.